Isolation and characterization of an estrogen-degrading *Pseudomonas putida* strain SJTE-1

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Abstract

In this report, *Pseudomonas putida* SJTE1 isolated from an enrichment culture of sludge was confirmed to degrade natural estrogens (17 β -estradiol, estrone, estriol), estrogenic chemicals (naphthalene and phenanthrene) and testosterone. The strain completely degraded 1 mg/L 17 β -estradiol in 24 h and transformed it into estrone; 90% and 75% of 50 mg/L and 100 mg/L 17 β -estradiol were utilized in 7 days, respectively. The transformation efficiency of this strain against natural estrogens was much higher than that against other estrogenic chemicals. Organic carbon sources, lipopolysaccharide and surfactants could enhance the degradation efficiency of strain SJTE-1 against 17 β -estradiol. The adsorption of 17 β -estradiol onto the biomass was the premise for transmembrane and cellular utilization of this chemical. This work has the potential to bioremediate the environmental estrogens.

Keywords Environmental estrogens · Biodegradation · *Pseudomonas putida* SJTE-1 · 17 β -Estradiol · Estrone

Introduction

Environmental estrogens (EEs) are considered one of the most important environmental contaminants which exist widely in different environments (Ting and Praveena 2017). Natural estrogens such as 17 β -estradiol (E2), estrone (E1) and estriol (E3), and the synthetic estrogen (17 α -ethinyl estradiol, EE2) are the major components of EEs. Some chemicals such as bisphenol A (BPA), nonylphenol (NP), anthracene, naphthalene and phenanthrene have also been

confirmed with different estrogenic activities (Tilghman et al. 2010; Zhang et al. 2016). Although their concentrations are very low (~ng/L) in the environment, they could lead to significant adverse effects on the reproductive system and physical metabolism of humans and wildlife (Yin et al. 2002; Huang et al. 2013; Zhang et al. 2011).

Microbial biodegradation is considered to be a promising technology for removing the environmental estrogens due to its high efficiency, low cost and no remaining secondary pollution (Combalbert and Hernandez-Raquet 2010; Yu et al. 2013; Ting and Praveena 2017). Some strains have also been identified to utilize estrogens as carbon sources or energy resources and transform them into harmless products under aerobic or anaerobic conditions. *Comamonas testosteroni*, *Rhodococcus zopfii*, *Nitrosomonas europaea*, *Sphingomonas* spp., *Sphingobacterium* spp. and *Pseudomonas* spp. were found to degrade the natural estrogens under aerobic condition; their degradation characteristics such as substrate spectrum, degradation efficiency and transformation periods varied considerably (Horinouchi et al. 2012; Yoshimoto et al. 2004; Shi et al. 2004; Yu et al. 2007; Masuda et al. 2007; Haiyan et al. 2007; Zheng et al. 2016). However, biodegradation of estrogens by heterotrophic bacteria is reported to be very slow, with the half-life of about 20–40 days under aerobic conditions and longer time would be expected under anaerobic conditions (Clouzot et al. 2008; Ying et al. 2003).

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Although the dissolved organic matter has been confirmed to facilitate the microbial transformation of E2 in an anaerobic aqueous environment, strains with higher degradation efficiency and shorter degradation period are still required (Gu et al. 2016, 2018).

Because of their great environmental tolerance and high degradation capability, *Pseudomonas* spp. strains have been applied in wastewater treatment to remove many organic pollutants such as bisphenol A (BPA), polycyclic aromatic hydrocarbon (PAH), persistent organic pollutants (POPs) and estrogens (Mita et al. 2015; Tay et al. 2014; Lee et al. 2003; Matsumura et al. 2009; Giese et al. 2007; Zeng et al. 2009; Zhang et al. 2016; Zheng et al. 2016). In this work, a Gram stain-negative strain SJTE-1 possessing high estrogen degradation efficiency was isolated and identified as *P. putida*; its characteristics were reported here.

Materials and methods

Chemicals and media

17 β -Estradiol (98% purity), estrone (99% purity), estradiol (97% purity), testosterone (98% purity) and 17 α -ethinylestradiol (99% purity) were purchased from Sigma-Aldrich (Allentown, PA, USA). Bisphenol A (99% purity), nonylphenol (NP, 99% purity), anthracene (99% purity), naphthalene (100% purity) and phenanthrene (99.5% purity) were purchased from AccuStandard, Inc. (New Haven, CT, USA). All chemicals were prepared as 10 mg/mL stock solutions in ethanol and stored at -20°C . Dansyl chloride, sodium carbonate, sodium bicarbonate, and sodium chloride (97% purity) were purchased from Sigma-Aldrich (Allentown, PA, USA). HPLC-grade formic acid (98.5% purity) was purchased from Alfa Aesar (Ward Hill, MA, USA). HPLC-grade methanol (99.9% purity), acetone (99.9% purity), hexane (97.5% purity) and ethyl acetate (97.5% purity) were purchased from Fisher Scientific (Philadelphia, PA, USA). HPLC-grade acetonitrile (99.5% purity) was purchased from EMD (Gibbstown, NJ, USA). All other chemicals were of analytical grade. Minimal medium (MM), pH 7.2 [containing (g/L) K_2HPO_4 , 0.1; $(\text{NH}_4)_2\text{HPO}_4$, 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02; FeCl_3 , 0.01; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1; NaCl , 0.1], was used for the isolation and enrichment of E2-degrading bacterium. Luria–Bertani (LB) medium [containing (g/L) tryptone, 10.0; yeast extract, 5.0; NaCl , 8.0] was used for subsequent culturing. For preparing solid plates, MM or LB medium was fortified with agar (15.0 g/L).

Strain isolation

Samples used for enrichment were collected from an active sludge of wastewater treatment plant of Shanghai, China (no specific permissions were required and this work did not involve any endangered or protected species). Approximately 10.0 g of the sludge sample was inoculated into 100 mL MM containing 10 mg/L E2 and was shaken at 180 rpm for 7 days at 30°C . A 10 mL enrichment culture was then inoculated into fresh 100 mL MM containing E2 and the incubation conditions were repeated. After three rounds of repeated enrichment, the culture was diluted and plated on the MM agar plates that had been pre-coated with 10 mg/L E2. Bacterial colonies with different morphologies that had grown on the plates were tested for their utilization capabilities of E2 as the sole carbon source. The strain with the fastest growth rate was isolated and designated as SJTE-1.

Identification of strain SJTE-1

A single colony of strain SJTE-1 was inoculated into LB medium and cultured at 30°C overnight. After the cells were centrifuged, DNA was extracted from the cell pellet and the 16S rRNA gene was amplified using the Bacterial 16S rDNA Kit (TaKaRa Biotechnology Co., Ltd. Dalian, China). The PCR procedure was performed as follows: denaturation at 94°C for 5 min, repeating denaturation at 94°C for 30 s 30 times, annealing at 55°C for 30 s and elongation at 72°C for 1.5 min, and final elongation at 72°C for 5 min. The PCR fragments were sequenced by Invitrogen Inc. (Shanghai, China), and the 16S rRNA gene sequence was deposited in GenBank (Accession no. JQ951925.1). The nearest relatives of strain SJTE-1 were identified by BLAST analysis of the 16S rRNA gene sequence against the National Center for Biotechnology Information (NCBI) 16S rRNA gene database and selected sequences were downloaded. A phylogenetic tree was constructed with MEGA 6.0 and the evolutionary distances were calculated with the Kimura two-parameter distance model. Un-rooted trees were built by neighbor-joining method with 1000 replications and maximum likelihood method (Saitou and Nei 1987). Morphological, physiological, and biochemical properties were tested as described in the Bergey's Manual of Determinative Bacteriology (Holt et al. 1994).

Growth analysis of strain SJTE-1 with different carbon sources

The growth of strain SJTE-1 cultured with different steroid chemicals and estrogenic chemicals (E2, E1, E3, EE2, testosterone, BPA, NP, naphthalene, phenanthrene and

anthracene) as the sole carbon source was detected using the Automatic Growth Curve Analyzer (BioScreen Testing Service, Inc., CA, US). Briefly, a single colony of strain SJTE-1 was inoculated into 20 mL LB medium and cultured overnight at 30 °C in a rotary shaker. After centrifugation, the cells were washed thrice with sterilized water and re-suspended in MM to form the inoculum about 1.0 OD₆₀₀. 10 µL of the cell pellet was inoculated into the 190 µL MM containing different estrogenic chemicals of different concentrations (1 mg/L, 10 mg/L, 25 mg/L, 50 mg/L and 100 mg/L) in the 500-µL well of 10×10 multi-well plate, and the initial OD₆₀₀ was 0.05. Wells containing cells without chemicals and those containing chemicals without inoculum were used as blank controls. The multi-well plates were incubated at 30 °C with constant shaking (180 r) for 7 days and the cell densities (OD₆₀₀) were detected every 30 min automatically. All experiments were repeated five times and the results were the average values with the errors.

Degradation characteristic analysis of strain SJTE-1 against estrogenic chemicals

The degradation efficiency of strain SJTE-1 against different chemicals was determined according to the loss of substrate. To analyze the degradation efficiency of strain SJTE-1 against E2, the cell inoculum was prepared as above, distributed into 1 L MM containing E2 of different concentrations (1 mg/L, 10 mg/L, 25 mg/L, 50 mg/L, and 100 mg/L), and cultured for 7 days at 30 °C in the shaker. The initial OD₆₀₀ was 0.05, and the flasks without cell inoculum were used as blank controls to assess the abiotic loss. Every 12 h, 5 mL of culture was taken out to determine the cell density and the E2 residues. High-performance liquid chromatography (HPLC) method was used for the residues detection of E2. The supernatant of each culture was pretreated by C18 SPE (solid phase extraction) columns (Waters Corporation, Milford, USA) and re-dissolved with acetonitrile after lyophilization. 10 µL aliquot was injected into a C18 column (Eclipse Plus C18; 5 µm, 4.6×150 mm, Agilent Tech., US) with water (15%) and methanol (85%) as mobile phase (1 mL/min) for 8 min. The UV wavelength was set at 254 nm with a diode array detector (DAD) and the column temperature was maintained at 30 °C. 0.1 mg/mL E2 dissolved in acetonitrile was used as the standard sample. Flow rate was kept at 0.7 mL/min. Five independent experiments were performed and results were expressed as the average values with errors. To analyze the metabolic intermediates of E2 transformed by strain SJTE-1, the strain was cultured in MM with E2 of 10 mg/L and detected every 24 h. The detection procedure of other estrogens (E1, E3, EE2, BPA, and NP) and naphthalene and phenanthrene was same as that of E2. Effect of different nutrient substances and metal ions on the degradation efficiency of strain SJTE-1 was determined by supplying them to the culture and detecting the residual

E2 after 24 h. Different substances (5 g/L of lactose, glucose, beef extract and tryptone), metal ions (1 mM NH₄NO₃, FeCl₂, FeCl₃, MnCl₂, CaCl₂, CuCl₂ and ZnCl₂) and surfactants (0.1% Tween 80 and lipopolysaccharides) were supplied to the MM with 10 mg/L E2, respectively.

Detection of E2 distribution in the biodegradation procedure

To track E2 distribution during the biodegradation process, the E2 residues in the supernatant and in the cell mass were detected at different times. Strain SJTE-1 was cultured in 1 L MM with 10 mg/L E2 at 30 °C for 7 days, and 5 mL of culture was taken out every 24 h; then the supernatants and the cell mass were separated by centrifugation. The supernatants were treated as above and resolved in acetonitrile. The cell mass was dissolved in acetonitrile before being filtrated with a 0.22-µm micro-filter. The E2 residues in the supernatant and in the solution of biomass were detected with HPLC. All experiments were performed five times and the results were the average values with errors.

Results

Isolation and identification of the estrogen-degrading strain SJTE-1

A strain was isolated after rounds of enrichment, which was rod shaped, Gram negative, having soft, ivory colored, flat and round colonies. Its optimal growth temperature was 30 °C and its growth pH ranged from 5.0 to 9.0. This strain could utilize E1, E2, E3, testosterone, naphthalene and phenanthrene as sole carbon sources. It was sensitive to DMSO, carbinol, and acetonitrile; ethanol was mild to this strain. This strain was designated as strain SJTE-1 and has been deposited to CGMCC with No. 6585.

The phylogenetic tree constructed showed that the 16S rDNA sequence of strain SJTE-1 (GenBank accession no. JQ951925.1) showed strong relationship with and high identity to those of *Pseudomonas* spp. (> 95%), especially *Pseudomonas putida* (> 99%) (Fig. 1). The evolutionary distance between strain SJTE-1 and *P. putida* was zero, and its evolutionary distance to *P. aeruginosa* was far (0.044). The farthest distance (0.298) was found between strain SJTE-1 and *Rhodococcus* (Fig. 1, Table S1). Therefore, strain SJTE-1 was a *P. putida* strain.

Strain SJTE-1 could utilize natural estrogens and estrogenic chemicals

Strain SJTE-1 was cultured with different steroids and estrogenic chemicals as its sole carbon source and cell growth was detected. When 1 mg/L and 10 mg/L

Fig. 1 Phylogenetic tree based on 16S rRNA gene sequences. NJ model was used and bootstrap analysis was performed with 1000 repetitions using MEGA 6.0. The bar indicates a genetic distance of 0.020

17 β -estradiol were used, strain SJTE-1 grew fast without obvious lag phase, and accumulated the largest biomass after 24 h (Fig. 2). Longer logarithmic phase and more biomass were observed when more E2 was supplied (Fig. 2a). This strain could also utilize E1, E3, TES, naphthalene and phenanthrene with longer lagging phase and less biomass (Fig. 2b–f), while it could not use

EE2, anthracene, BPA and NP as its sole carbon source. Although their degradation trends were similar, the utilization preference of strain SJTE-1 to these chemicals was E2 > E1 > E3 $\approx$ TES > naphthalene > phenanthrene. Compared with other reported strains, strain SJTE-1 had stronger tolerance capability and wider substrate spectrum (Horinouchi et al. 2012; Yoshimoto et al. 2004; Shi et al.

Fig. 2 Growth curve of strain SJTE-1 in minimal medium with different carbon sources. The initial concentration of different chemicals ranged from 0 to 100 mg/L. **a–f** Growth curves of strain SJTE-1 cultured with E2 (**a**), E1 (**b**), E3 (**c**), testosterone (**d**), naphthalene (**e**)

2004; Yu et al. 2007; Masuda et al. 2007; Haiyan et al. 2007).

Strain SJTE-1 could degrade 17 β -estradiol efficiently and estrone was the first intermediate

The degradation efficiency of strain SJTE-1 against different estrogenic chemicals was detected and is illustrated in Fig. 3. The standard curve of each chemical detected by the HPLC method was plotted; the degradation efficiency

and phenanthrene (**f**) as sole carbon sources. Cells were cultured for 7 days and samples were detected every 12 h. Biomass is shown as OD₆₀₀; the mean and standard deviation were calculated from five independent experiments

of strain SJTE-1 against each chemical was calculated by dividing the input amounts of this chemical with its residual amounts or by measuring the amount of generated product. Results showed that substrate consumption followed biomass accumulation. Strain SJTE-1 could degrade 1 mg/L 17 β -estradiol completely or 90% of 10 mg/L 17 β -estradiol in 24 h, and it could degrade 90% of 50 mg/L E2 in 6 days; about 25% of 100 mg/L E2 was still detectable after 7 days (Fig. 3a). The degradation period of strain SJTE-1 against other estrogenic chemicals was longer.

Fig. 3 Degradation efficiency of strain SJTE-1 against different chemicals. The initial concentration of estrogenic chemicals was 0–100 mg/L. **a–f** Degradation efficiency plots of strain SJTE-1 against E2 (**a**), E1 (**b**), E3 (**c**), testosterone (**d**), naphthalene (**e**) and phenanthrene (**f**). The strain was cultured for 7 days and the residual

chemicals in the cultures were detected every 12 h with HPLC. The efficiency was calculated from the ratio of the chemical's residue to its initial input; the mean and standard deviation were calculated from five independent experiments

72 h and 120 h were needed to degrade 10 mg/L estrone and E3 completely, respectively; only half of 100 mg/L phenanthrene was degraded in 7 days (Fig. 3b–f). In addition, metal ions and nutrient supplements could facilitate the utilization of estrogens differently. Metal ions had very little influence, while tryptone improved the degradation efficiency of strain SJTE-1 by 10% (Table S2). It was probably because these organic chemicals could trigger faster cell growth at the initial stage producing more biomass and more electron receptor to facilitate the utilization of estrogens (Gu et al. 2016, 2018). Tween 80 and lipopolysaccharide also promoted the strain's degradation

efficiency a little; probably it could improve the solubility of estrogenic chemicals and facilitate their transportation into cells (Table S2). Further analysis of the E2 metabolite showed that E1 was detected in culture after 12 h and the increase of E1 was accompanied by the reduction of E2. When E2 was not detectable in culture, E1 was still on a relatively stable concentration, and the accumulated E1 was degraded to non-estrogenic chemicals in 2 days (Figs. 4, 5). Therefore, strain SJTE-1 degraded E2 efficiently, with stronger degradation capability and shorter degradation period compared to previous reports (Horinouchi et al. 2012; Shi et al. 2004; Yu et al. 2007).

Fig. 4 Metabolite analysis of strain SJTE-1 against E2. Strain SJTE-1 was cultured with 10 mg/L E2 as the sole carbon source and the cultures were detected with HPLC. The standard of E2 and E1 was used (A and B); and the cultures were detected at two time points 72h (C) and 144 h (D). The whole elution time was 8 min; the peak of E1 (elution time is 5.6 min) and the peak of E2 (elution time is 3.9 min) were marked

Fig. 5 Concentration of E1 and E2 in the biodegradation process. The E2 concentration (square) and E1 abundance (triangle) during the culture of strain SJTE-1 were detected with HPLC. The initial concentration of E2 was 10 mg/L. Error bars represent the average deviations

E2 distribution during the degradation process

The E2 residues in culture and in cell mass were determined to track the distribution of E2 in the biodegradation procedure. Results showed that after 24 h, over 80% of 10 mg/L E2 was degraded and the biomass reached the maximum. The cell proliferation and the E2 degradation were the fastest in 24 h, while in the stationary phase, the E2 reduction speed slowed down (Fig. 6). At first, E2 was distributed in culture, 6 h later, the adsorbed E2 on biomass was nearly 4 mg/L. In the next 90 h, the cell-adsorbed E2 reduced and was 1 mg/L. When E2 in culture could not be detected, the E2 adsorbed on biomass also declined (Fig. 6). These results implied that the adsorption of estrogens on biomass was probably the premise of its transmembrane transportation and cellular degradation. The E2 molecules adsorbed onto cells and those transported into cells could keep a balance and support cell growth. The E2 in culture decreased with the accumulation of biomass; along with the E2 degradation, generated E1 molecules were secreted into the culture. At the end of

Fig. 6 E2 residues in culture and in cells in the biodegradation process. E2 concentration in solution and in cells was detected and the biomass is also shown. The initial concentration of E2 was 10 mg/L. Error bars represent average deviations

the stationary phase, E2 was almost degraded completely, undetectable in culture and on biomass (Fig. 7).

Discussion

Natural estrogens, synthetic estrogens and other estrogenic chemicals have been recognized as potential endocrine disruptors. Although their concentration in environment is normally at about ng per liter levels, field and laboratory studies have demonstrated that they can still alter normal hormone functions and the physiological status of wildlife (Liu et al. 2011, 2012; Huang et al. 2013, 2015; Ting and Praveena 2017). Biodegradation has been considered as the efficient and predominant method to remove these chemicals and remediate the polluted environment. However, it has been shown that the biodegradation of estrogens by heterotrophic bacteria is very slow, with a biodegradation half-life of about 20–40 days under aerobic conditions, and longer time was expected under anaerobic conditions (Clouzot et al. 2008; Ying et al. 2003). Different strategies have been used to enhance the efficiency and shorten the period. For example, dissolved organic matter could improve biodegradation efficiency of E2 in an anaerobic aqueous environment by serving as an electron shuttle mediator (Gu et al. 2016, 2018). Combination of photodegradation and biodegradation methods could also accelerate estrogen removal in natural aquatic environment (He et al. 2018). Biochar was also used to reduce steroid hormone pollution from poultry and swine manure in sandy soil (Alizadeh et al. 2018). In any event, the

microorganisms with great degradation capability and short degradation period are still expected.

Pseudomonas spp. have been widely applied in pollutant removal due to their outstanding environmental tolerance and great degradation efficiencies. *Pseudomonas putida* OUS82 and G7 could utilize naphthalene (Tay et al. 2014; Lee et al. 2003); *P. putida* KA4 and KA5 were confirmed with high BPA biodegradation abilities (Mita et al. 2015; Matsumura et al. 2009). Some *Pseudomonas* strains found in the wastewater treatment were responsible for estrogen biodegradation (Weber et al. 2005; Pauwels et al. 2008; Zheng et al. 2016). In this work, the isolated *P. putida* SJTE-1 could degrade 1 mg/L 17 β -estradiol completely in 24 h and 90% of 17 β -estradiol (50 mg/L) in 7 days. Compared to other reported estrogen-degrading strains, strain SJTE-1 had greater degradation efficiency and shorter degradation period (Haiyan et al. 2007; Horinouchi et al. 2012; McAdam et al. 2010; Shi et al. 2004; Weber et al. 2005; Yoshimoto et al. 2004; Yu et al. 2007). For example, *Rhodococcus* sp. and *Sphingomonas* sp. degraded 50% of 0.8 mg/L E2 in 24 h and 90% of E2 in 120 h (Futoshi et al. 2010); *N. europaea* degraded E1 and E2 with degradation rate constants of 0.056 h⁻¹ for E1 and 1.3 h⁻¹ for E2 (Shi et al. 2004). As environmental estrogens are always in trace concentration, some strains may lose their degradation capability in such condition (McAdam et al. 2010). Strain SJTE-1 was also confirmed to be able to keep a stable degradation capability even under low concentration of estrogens (~10 ng/L) (data not shown). It could also utilize several estrogens and estrogenic chemicals with different efficiencies, probably due to the variation in the core structure and the side chain groups.

As water solubility of most estrogens and estrogenic chemicals was poor, a large amount of biofilm was produced to enhance their solubility and facilitate their utilization when strain SJTE-1 was cultured in MM containing estrogens. The matrix structure and the lipopolysaccharide components of biofilm were speculated to serve the estrogen adsorption, enhance the solubility, and fasten the transmembrane transportation. The supplied lipopolysaccharides and Tween 80 actually enhanced the degradation efficiency of strain SJTE-1 against E2. Besides, biofilm has been proved to help cells attach to activated sludge and contribute to its on-site degradation process in real environment. Meanwhile, supplied carbon sources could also enhance the aerobic degradation of strain SJTE-1 against E2, consistent with the previous reports in anaerobic conditions (Gu et al. 2016, 2018). Therefore, *P. putida* SJTE-1 possessed great biodegradation characteristics; its finding could enrich the bacterial sources for estrogen degradation and promote related biodegradation mechanism study.

Fig. 7 The adsorption and degradation model of strain SJTE-1 against E2. 1–7 represent different growth stages of strain SJTE-1. Black balls represent the E2 molecules and grey ones represent E1 molecules

1-2: Logarithmic Phase;
3-6: Stationary Phase;
7: Decline Phase

Author contributions RL designed the experiments and wrote the manuscript. DZ and PW performed the experiments. XW assisted the experiments. All the authors discussed the results and commented on the manuscript.

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Availability of data and material The data supporting the conclusions of this article are included within the article and additional file. The estrogen-degrading *P. putida* SJTE-1 has been deposited to CGMCC with No. 6585.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval and consent to participate This article does not contain any studies with human participants or animals performed by any of the authors.

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